Communications to the Editor

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CO-OCCURRENCE AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF THE GLYCOSIDES OF RHODEASAPOGENIN AND ITS ANALOGS WHICH DIFFER IN THE F-RING STRUCTURE

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Two spirostanol glycosides (steroid saponins) R-3 (I) and R-8 (II), which were regarded, respectively, as pure 1-O-α-L-rhamnopyranosyl-(1-2)-β-D-xylopyranoside and 3-O-β-D-glucopyranoside of rhodeasapogenin(25S) (III), were shown by ^{13}C-NMR to be accompanied by the corresponding glycosides of isorhodeasapogenin(25R) (IV) and convallamarogenin($\Delta^{25(27)}$) (V). Qualitative and preparative separation by HPLC of the components of I and II were studied and successfully achieved by a combination of two methods. They consisted equally of not three but four compounds, which were isolated in the pure state. Three of them were identified as the glycosides of III (major), IV and V, while the fourth was considered to have the same sugar moiety as I or II combined with a new aglycone different from III - V in the F-ring structure.

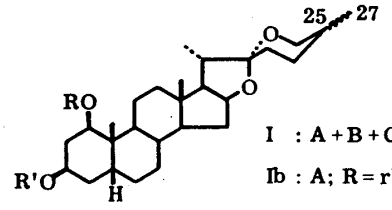
The methods were applied also to some commonly known sterod saponins.

KEYWORDS —— steroid saponin; rhodeasapogenin glycoside; 25R-, 25S-, 25(27)-dehydro-spirostanol glycoside; ^{13}C-NMR; RP-HPLC

Existence, sometimes co-occurrence, of the natural spirostanols which are different from each other only in the structure at C-25 (25R, 25S, $\Delta^{25(27)}$) is well known.[1a] This suggests co-occurrence also of their glycosides (steroid saponins) with the same sugar moiety.[2]

In such a case, qualitative and preparative separation of the components is hardly possible at present, in contrast to the case of a mixture of free spirostanols.[3]

During our study on the constituents of the underground parts of Rhodea japonica, two steroid saponins R-3 (I),[4] colorless[5] needles (Me_2CO), mp 243-244°C,[6] $[\alpha]_D$-99.2°,[7] and R-8 (II),[8] needles (dil.MeOH), mp 260-264°C, $[\alpha]_D$-61.7°, were obtained. They were homogeneous in usual TLC and reversed-phase (RP) HPTLC using various solvent systems, were considered to have a 25S-spirostanol as the aglycone on the basis of their IR[9] and ^{1}H-NMR[10] spectra, and were characterized, respectively, as 1-O-α-L-rhamnopyranosyl-(1-2)-β-D-xylopyranoside (rha-xyl) and 3-O-β-D-glucopyranoside (glc) of



A= 
B= 
C= 

I : A+B+C; R=rha—(2)xyl, R'=H
Ib : A; R=rha—(2)xyl, R'=H
Ic : B; R=rha—(2)xyl, R'=H
Id : C; R=rha—(2)xyl, R'=H

II : A+B+C; R=H, R'=glc
IIb : A; R=H, R'=glc
IIc : B; R=H, R'=glc
IId : C; R=H, R'=glc

III : C; R=R'=H
IV : B; R=R'=H
V : A; R=R'=H

25S,5β-spirostane-1β,3β-diol (rhodeasapogenin) (III). However, the aglycone yielded by acid hydrolysis of I or II was shown in ^{13}C-NMR[11] to be a mixture of III, its 25R-epimer (isorhodeasapogenin) (IV) and the 25(27)-dehydro derivative (convallamarogenin) (V).

When the ^{13}C-NMR spectra of I and II, intact, (Fig.1) were taken and the data were compared with those of III, IV and V (Table I), their unhomogeneities were apparent, and the signals due to carbon atoms of the sugar moieties[12] and of A-rings of aglycones were consistent with the given structures I and II. Thus, ^{13}C-NMR is of use for purity control of a steroid saponin and quantitative[13] determination of the contaminants if any.

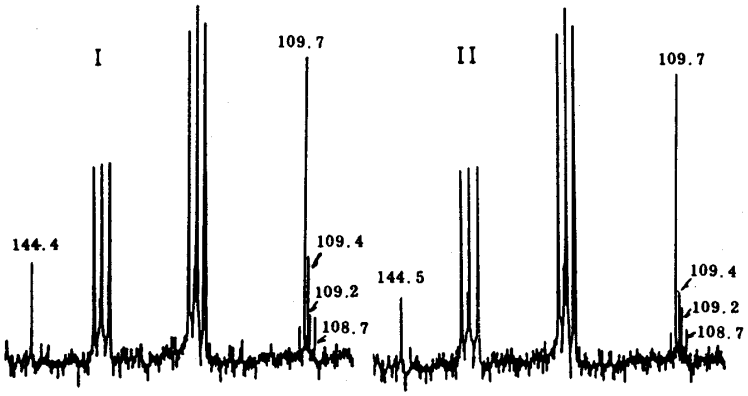


Fig. 1. ^{13}C-NMR Spectra of I and II (δ in C_5D_5N, 25.0 MHz, at 28°C)

Table I. Comparison of ^{13}C-NMR Data

C No.	III(25S)	IV(25R)	V($\Delta^{25(27)}$)
20	42.5	42.0	42.0
21	14.9	15.0	15.0
22	109.7	109.2	109.4
23	26.4	31.9	29.0[a]
24	26.2	29.3	33.3[a]
25	27.5	30.6	144.5
26	65.1	66.9	65.1
27	16.3	17.3	108.7

a: may be reversed.

This method, however, needs a rather large sample or a long run of FT measurement. Preparative isolation of the individual components is another problem. Therefore, in order to find a new means requiring much less sample and being useful for the preparative purpose as well, application of high-performance liquid chromatography (HPLC) was studied.

There are several papers on HPLC resolution of spirostanols[3a] and of their glycosides,[1b,14] but none of a mixture of the glycosides, of which aglycones are different only in the structures at C-25, has so far been reported except one related communication which deals with separation of neopentologenin(25S) 2-O-xyloside peracetate from a contaminant, the 25(27)-dehydro congener.[14b] Taking this into account, the qualitative separation of I and II into their components by RP-HPLC was tried, and both were found to give three peaks (a), (b) and (x) (Fig.2) under the following condition(method A); instrument, TWINCLE and TRIROTAR-III (JASCO) equipped with a refractive index detector SHODEX RI SE-11 (Showa Denko); column, Radial Pak C-18 (10μ) (i.d., 8 mm; length, 100 mm)(Waters); solvent for sample, DMF (concentration, 1 - 2%); eluent, 80 - 90% MeOH; flow rate, 0.5 - 1.0 ml/min; amount of sample loaded, 0.1 - 1.0 mg; at room temperature. The method was satisfactorily extended to preparative separation of 10 - 30 mg of a sample (5 - 20% solution in DMF) at one time. Since the three peaks were presumed to correspond to the glycosides of III, IV and V, their isolation was then carried out.

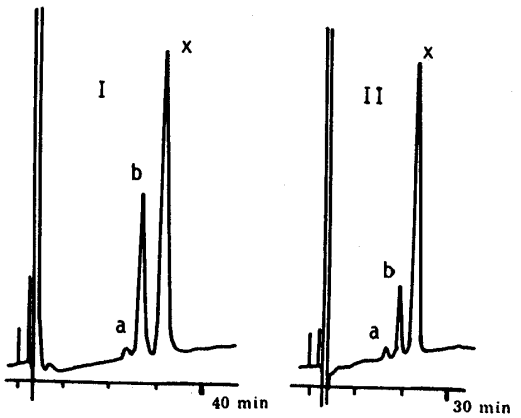


Fig. 2. HPLC of I and II
Condition: sample loaded, 1.0 mg (5% in DMF); eluent, 80% MeOH; flow rate, 1.0 ml/min.

By repeated HPLC A, I (430 mg) and II (150 mg) provided, respectively, three glycosides: I_a,

prisms (Me_2CO-MeOH-water) (12 mg), mp 275-277°C, $[\alpha]_D$-51.4°(c=0.42); I_b, needles (MeOH) (52.3 mg), mp 287°C, $[\alpha]_D$-97.6°; I_x (200 mg) and II_a, needles (dil.MeOH) (3.3 mg), mp 257-259°C, $[\alpha]_D$-31.2° (c=0.17); II_b, plates (dil.MeOH) (14.6 mg), mp 270-274°C, $[\alpha]_D$-74.0°($CHCl_3$-MeOH (1:1)); II_x (98.6 mg). I_b and II_b were proved by ^{13}C-NMR[11,12] to be pure 1-O-rha-xyl and 3-O-glc of V, while I_x and II_x were found, against an expectation, to consist of the glycosides of III and IV. The spectra of I_a and II_a were the same as those of I and II in regard to the sugar moiety, and the signals due to C-20 - C-27 of the aglycones were identical to each other but different from those of III, IV and V, suggesting a modified F-ring. Their structures will be discussed in a succeeding paper.

Further separation of I_x or II_x into III- and IV-glycosides was then attempted by RP-HPLC using eight kinds of columns and various eluents at room temperature. Almost all experiments failed. Only when a TSK-GEL LS-410 column (5μ) (i.d., 4.6 mm; length, 250 mm) (Toyo Soda) was used (solvent for sample, DMF; eluent, 90% MeOH; flow rate, 0.5 ml/min), was a resolution slightly observed (Fig.3). Subsequently, in consideration of the relation between performance and column temperature,[15] it was found that by elevating the temperature (20 → 60°C) the retention volumes (VR) of the components decreased and the resolution got worse (showing a single peak at 60°C) and that by lowering the temperature (20 → -20°C) the eluent pressure and VR increased and as a result the components were clearly separated.[15a] Eventually, the following condition (method B) was employed for the resolution of I_x or II_x: column, TSK-GEL LS-410 (as mentioned above); column temperature, 0°C; solvent for sample, DMF (concentration, 2 - 5%); eluent, 70 - 90% MeOH; flow rate, 0.2 -0.5 ml/min; amount of sample loaded, 0.1 - 0.5 mg. (Fig.4). The above column is for qualitative use, but a much larger amount (10 - 30 mg) of sample (5 - 20% solution in DMF) could also be loaded. Thus, I_x (40 mg) and II_x (32.9 mg) gave I_c, needles (pyridine - MeOH) (10.7 mg), mp 244°C, $[\alpha]_D$-97.7°; I_d, needles ($CHCl_3$-MeOH-water) (27.2 mg), mp 262°C, $[\alpha]_D$-104.6° and II_c, needles (dil.MeOH) (7.5 mg), mp 260-265°C, $[\alpha]_D$-58.3°; II_d, needles (dil.MeOH) (13.2 mg), mp 275-280°C, $[\alpha]_D$-64.5°. I_c and II_c were identified by ^{13}C-NMR,[9,10] as pure 1-O-rha-xyl and 3-O-glc of IV, while I_d and II_d as those of III, respectively.

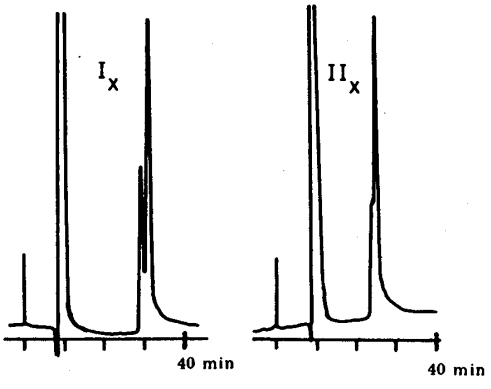


Fig. 3. HPLC of I_x and II_x at 25°C
Condition: sample loaded, 0.2 mg (5% in DMF).

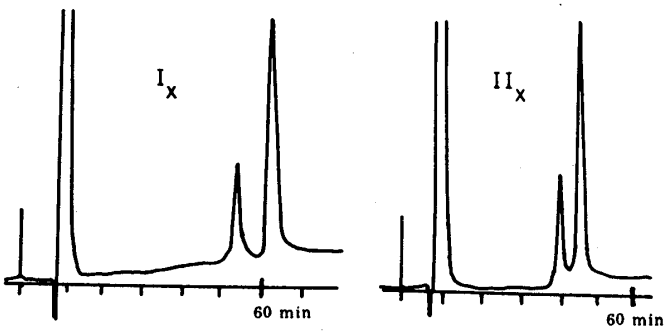


Fig. 4. HPLC of I_x and II_x at 0°C
Condition: sample loaded, 0.2 mg (5% in DMF); eluent, 90% MeOH; flow rate, 0.5 ml/min.

A number of steroid saponins have been reported with their mps and $[\alpha]_D$ values,[1b] but some of them possibly might be contaminated with steroid saponins of which aglycones are the 25-epimer, the 25(27)-dehydro derivative and a new congener. Combination of the present two HPLC methods is worth special mention not only as a useful means for qualitative[16] and preparative separation of the four co-existing analogs of I or II, but also as a clue to new technique, by slight modification if necessary, for purity control and isolation in the pure state of steroid saponins in general.

Diosgenin(25R) (VI) 3-O-chacotrioside (dioscin) (VII), plates (dil.MeOH), mp 296-298°C, $[\alpha]_D$ -106.6°, from the rhizomes of Dioscorea septemloba[17] showed a single peak in the HPLC A and B, while

"dioscin", plates (dil.MeOH) (200 mg), mp 290-294°C, $[\alpha]_D$-110°, from D.tokoro (purified by column chromatography on silica gel),[18] being homogeneous in usual TLC, RP-HPTLC and HPLC A, provided by HPLC B VII (135 mg),[19] mp >300°C, $[\alpha]_D$-100°; and 3-O-chacotrioside of the 25S-epimer (yamogenin) of VI, needles (dil.MeOH) (36 mg), mp >300°C, $[\alpha]_D$-120.9°. Both of these were identified as such by ^{13}C-NMR. Gracillin,[17,20] plates (dil.MeOH), mp 299-301°C, $[\alpha]_D$-97.8°, and F-gitonin,[21] needles (dil.BuOH), mp 251-254°C, $[\alpha]_D$-59.8°, gave, respectively, a single peak in the HPLC A and B. Seemingly (TLC) pure digitonin (3-O-digitopentaoside of digitogenin(25R) (VIII)),[22] plates (dil. BuOH), mp 259-263°C, $[\alpha]_D$-53.7°, obtained from a commercial "digitonin" (Merck) by chromatographies on silica gel and RP-8 (Merck) columns followed by recrystallization, was shown by HPLC B to be accompanied by a minor saponin (about 11%),[16] which was presumed[2b] to be the 25S-epimer of VIII.

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7) All optical rotations were taken with a JASCO DIP-SL automatic polarimeter (cell= 1 dm) at 18-28°C, and unless otherwise specified, in a pyridine solution (c= 0.9 - 1.2).
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